Demonstration of New Surface Antigens in Cells Transformed by Papovavirus SV40 by Cytotoxic Tests.* (30561)

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Hamster cells transformed by papovavirus SV40 acquire new antigens unrelated to the viral capsid antigens. These antigens have been demonstrated indirectly by transplantation rejection procedures(1-4) and directly by complement fixation(5-7) and immunofluorescent technics(8-10). The latter method has located one of the antigens in the nucleus (8-10) and it appears probable that the complement-fixing and immunofluorescent antigens are identical(7,10).

Recently the presence of new surface antigens was demonstrated in cells transformed by SV40(11); sera from hamsters vaccinated with the homologous virus and resisting the transplantation of cells transformed by SV40 reacted with these surface antigens in indirect immunofluorescent tests. This report describes the preparation of specific cytotoxic rabbit antibodies directed against the virus-transformed cells.

Materials and methods. Cell lines. The H-50 cells were derived from a hamster tumor induced by SV40(12). The cells have undergone 90-100 passages in tissue culture and are free from infectious SV40(13). These cells synthesize the SV40 intranuclear tumor antigen(8) and the specific surface antigens previously described(11).

Other cell types used were hamster embryo fibroblasts (HEF), the BHK21 cell line(14), and cells from a hamster tumor induced by adenovirus type 7. Cells from the hamster tumor induced by adenovirus type 7 and BHK21 cells do not contain the intranuclear SV40 tumor antigen synthesized by cells transformed by SV40 and animals vaccinated with SV40 do not resist transplantation of BHK21 cells(15).

All the cell types were grown in 16 oz prescription bottles in Eagle's medium supplemented with 10% heat inactivated calf serum, 100 units of penicillin and 100 μg of streptomycin per ml.

Preparation of antigen. The antigen for immunization of rabbits was prepared from H-50 cells. The cells, grown in 16 oz prescription bottles, were washed 6 times with Tris buffer (pH 7.4) and resuspended with the help of a rubber policeman in Tris buffer at a concentration of 6-8 $\times$ 10^6 cells/ml. The cells in suspension were ruptured by sonication for 30 seconds at 10 KC/second. The sonicated material was centrifuged at low speed and the supernatant fluid was used to immunize the rabbits.

Preparation of antiserum. White cottontail rabbits were used for preparation of anti-H-50 cell serum. The animals received 3 subcutaneous injections of 1 ml of H-50 cell extract mixed with 1 ml of complete Freund's adjuvant. The injections were given at 10-day intervals. Thirty days after the last injection, animals were given 1 ml of the H-50 cell extract without Freund's adjuvant. The animals were bled 10 days after the last injection, and the sera were stored at —20°C without any preservative.

Cytotoxic test. The modified cytotoxic test described by Bennett *et al*(16) was used. The cells from 16 oz bottles were dispersed by 0.2% trypsin, washed in Eagle's medium without calf serum, centrifuged and resuspended at a concentration of approximately 10^6 cells/ml. Serial 2-fold dilutions of antiserum were prepared in Eagle's medium without calf serum. Equal parts (0.2 ml) of cell suspension (1 $\times$ 10^6 cells/ml) were added to the serum dilutions. After 30 minutes of incubation at room temperature, 0.2 ml of undiluted guinea pig serum as a source of complement was added to each tube (unless otherwise indicated) and incubation was continued at 37°C for 45 minutes. At the end of incubation period, 0.2 ml of 0.2% trypan blue in Eagle's medium without calf

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TABLE I. Cytotoxic Reaction of Anti-H-50 Rabbit Serum Against H-50 and Normal Cells in Presence of Complement.*

Test serum	Cells used for absorption	Cells in test	Serum dilution 1/10	1/20	1/40	1/80	1/160	1/320	Cytotoxic titer
			% Cells stained by trypan blue						
Anti-H-50 rabbit serum									
No. 1	none	H-50	100	100	100	92	70	21	1/160
2	"	"	100	100	100	68	75	4	1/160
1	HEF	H-50	100	100	100	84	54	8	1/160
2	"	"	96	94	77	41			1/80
1	none	HEF	94	100	91	82	5		1/80
2	"	"	100	88	14				1/40
1	HEF	"	55	9	8	7			1/10
2	"	"	5	3					<1/10

* Guinea pig serum was used as a source of complement. HEF = normal hamster embryo fibroblasts.

serum was added to each tube. Cell viability was determined by calculating the % of dead cells (those stained by trypan blue). The cytotoxic titer was expressed as that dilution of antiserum in which the percentage of dead cells was closest to 50%. Included in each cytotoxic test were a known positive and negative serum. Cells were also incubated in the presence and absence of complement without additional serum.

Absorption of the anti-H-50 rabbit serum was carried out where indicated by diluting the serum 10-fold with Tris buffer (pH 7.4) followed by addition of about 10^8 HEF cells. The mixture was incubated at 37°C for 1 hour and was then further incubated at 4°C overnight. The cells were removed by slow speed centrifugation.

Results. Table I shows the results of representative cytotoxic tests with 2 anti-H-50 rabbit sera. The cytotoxic titer of the unabsorbed sera is 1/160 against H-50 cells in the presence of guinea pig complement. The cytotoxic titer of serum no. 1 against H-50 cells after absorption with HEF remained at 1/160 whereas the titer of serum no. 2 against the same cells was 1/80 after absorption with HEF. Both sera after absorption with HEF cells showed very low cytotoxic titers against the homologous cells; this indicates that the antibodies to normal cell surface antigens were successfully removed by absorption with the HEF cells.

Dependence of the reaction on complement is demonstrated in Table II. The cytotoxic titers of the sera dropped from 1/160 and 1/80 in the presence of complement to 1/10 and less than 1/10 in the absence of complement. The necessity of participation of complement for the cytotoxic reaction has been confirmed in numerous experiments using various sources of complement.

Table III summarizes the affinity of absorbed anti-H-50 rabbit sera for the surface antigens of H-50 cells. The cytotoxic titers of the anti-H-50 rabbit sera after absorption with HEF were very low against cells not transformed by SV40 when compared to titers against H-50 cells. The titers against BHK21 cells, HEF cells, and cells from a hamster tumor induced by adenovirus type 7 are less than 10 or 1/10 after absorption with HEF cells whereas the titers against H-50 cells are 1/80 and 1/160. Absorption with H-50 cells decreased the titer from 1/160 to <1/10. These results indicate that the cytotoxic test is detecting antibodies specifically directed against surface antigens of H-50 cells.

Pre-inoculation serum from rabbits immunized with H-50 cells had no cytotoxic effect

TABLE II. Effect of Guinea Pig Complement on the Cytotoxic Reaction.

Test serum	Cells used for absorption	Cells in test	Guinea pig complement	Cytotoxic titer
Anti-H-50 rabbit serum				
No. 1	HEF	H-50	Present	1/160
2	HEF	H-50	"	1/80
1	HEF	H-50	Absent	1/10
2	HEF	H-50	"	<1/10

TABLE III. Specificity of Rabbit Cytotoxic Antibody for H-50 Surface Antigens.

Test serum	Cells used for absorption	Cells in test	Cytotoxic titer
Anti-H-50 rabbit serum			
No. 1	none	H-50	1/160
1	HEF	"	1/160
2	none	"	1/160
2	HEF	"	1/80
1	none	HEF	1/80
1	HEF	"	1/10
2	none	"	1/40
2	HEF	"	<1/10
1	none	BHK21	1/80
1	HEF	"	1/10
1	"	Adeno 7 —t.c.*	1/10
2	"	"	1/10
1	HEF + H-50	H-50	<1/10
Normal rabbit serum	none	"	<1/10
Anti-normal bovine serum rabbit serum	"	"	<1/10

* t.c.—tumor cells.

on H-50 cells in the presence of guinea pig complement (Table III). Serum against normal bovine serum prepared in rabbits also had no effect on H-50 cells in the presence of complement. Similarly, complement alone had no cytotoxic effect on H-50 cells or other cells used in this study.

Discussion. The results of this study using heterologous serum absorbed with normal cells has demonstrated that hamster cells transformed by SV40 possess new specific surface antigens. Inability of serum absorbed with HEF to react not only with the homologous cells but with the BHK21 cells and adenovirus type 7 hamster tumor cells supports the specificity of the reaction with the H-50 cells. Complete removal of cytotoxic activity against H-50 cells following absorption with the homologous cells confirms the distinctness of the H-50 surface antigens.

The cytotoxic reaction in this system is dependent on the presence of complement. Cytotoxicity is, therefore, due to an antigen-antibody reaction, presumably taking place at the cell surface, which binds the complement. This is similar to the observation of Bases(17) in a polyoma system using heterologus rabbit serum and loss of ability to form colonies as criteria for reactivity. Cytotoxic tests have also been widely used to characterize cells from murine leukemia (see (18) for recent review). Cells in tissue culture have been shown to absorb serum proteins from the medium in which they are cultivated(19) but this type of reaction does not seem to affect the specific reaction in the SV40 system, because no cytotoxicity was observed when rabbit serum against normal bovine serum was reacted with H-50 cells in the presence of complement.

These results extend the observation that cells transformed by SV40 possess new antigens as demonstrated by transplantation rejection procedures(1-4) and by indirect immunofluorescent tests(11) using hamster serum from animals rendered resistant to transplantation by vaccination with SV40. The latter technic as well as the results reported here, place these antigens at the surface of the cell. This is also suggested by the finding that irradiated, viable, but non-dividing SV40 tumor cells can prevent induction of tumors by SV40 virus(20) and by suggestive evidence using ruptured cells that point in the same direction(1). These surface antigens are distinct from the SV40 intranuclear tumor antigen because hamster sera reacting with the tumor antigen fail to react with the surface antigen(8) and sera containing antibody against the surface antigen fail to react with the tumor antigen.

Attempts to detect surface antigens by indirect immunofluorescent methods using the cytotoxic rabbit sera have thus far been unsuccessful because of failure to remove non-specific staining by absorption. These tests have revealed, however, that the sera do not react with the intranuclear SV40 tumor antigen. This interpretation is possible because the non-specific reactions do not involve the nucleus. Hamster sera reacting with the surface antigen of the transformed cells(11) do not appear to be cytotoxic although high concentrations prevent the growth of the cells. The most probable explanation for the result is that the antibody level of the hamster sera is low but our present data do not rule out the possibility that the hamster and rabbit sera are reacting with different antigens at the surface of the transformed cells.

Direct evidence that synthesis of the surface antigens is under control of the virus genome and the role of these antigens in tumor immunity or in the neoplastic process itself is lacking, but these problems are under investigation.

Summary. Immunization of rabbits with cells transformed by papovavirus SV40 resulted in the production of cytotoxic antibodies specific for the homologous cells. Following absorption with normal hamster embryo fibroblasts, these sera failed to react with normal hamster cells, with BHK21 hamster cells, or with hamster cells transformed by adenovirus 7. Absorption with the homologous SV40 transformed cells removed all activity. The reaction observed was dependent on complement. The results suggest the presence of new specific antigens at the surface of cells transformed by SV40 and support the previous localization of surface antigens by the indirect immunofluorescent method.

ADDENDUM. Since this work was completed, we have observed that absorption of the rabbit antisera with sheep erythrocytes decreases, but does not abolish, the cytotoxic reaction against the H-50 cells.

1. Khera, K. S., Ashkenazi, A., Rapp, F., Melnick, J. L., J. Immunol., 1963, v91, 604.

2. Habel, K., Eddy, B. E., Proc. Soc. Exp. Biol. and Med., 1963, v113, 1.

3. Koch, M. A., Sabin, A. B., ibid., 1963, v113, 4.

4. Defendi, V., ibid., 1963, v113, 12.

5. Black, P. H., Rowe, W. P., Turner, H. C., Huebner, R. J., Proc. Nat. Acad. Sci., 1963, v50, 1148.

6. Kitahara, T., Butel, J. S., Rapp, F., Melnick, J. L., Nature, 1965, v205, 717.

7. Habel, K., Jensen, F., Pagano, J. S., Koprowski, H., Proc. Soc. Exp. Biol. and Med., 1965, v118, 4.

8. Rapp, F., Butel, J. S., Melnick, J. L., ibid., 1964, v116, 1131.

9. Pope, J. H., Rowe, W. P., J. Exp. Med., 1964, v120, 121.

10. Gilden, R. V., Carp, R. I., Taguchi, F., Defendi, V., Proc. Nat. Acad. Sci., 1965, v53, 684.

11. Tevethia, S. S., Katz, M., Rapp, F., Proc. Soc. Exp. Biol. and Med., 1965, v119, 896.

12. Ashkenazi, A., Melnick, J. L., J. Nat. Cancer Inst., 1963, v30, 1227.

13. Melnick, J. L., Khera, K. S., Rapp, F., Virology, 1964, v23, 430.

14. Macpherson, I., Stoker, M., ibid., 1962, v16, 147.

15. Rapp, F., Khera, K. S., Melnick, J. L., Nature, 1964, v201, 1349.

16. Bennett, B., Old, L. J., Boyse, E. A., Transplantation, 1964, v2, 183.

17. Bases, R., Cancer Res., 1964, v24, 1216.

18. Old, L. J., Boyse, E. A., Ann. Rev. Med., 1964, v15, 167.

19. Hamburger, R. N., Pious, D. A., Mills, S. E., Immunology, 1963, v6, 439.

20. Goldner, H., Girardi, A. J., Larson, V. M., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1964, v117, 851.

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Induction of Implantation in the Hypophysectomized Rat with Gonadotrophins.* (30562)

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Implantation of the rat blastocyst takes place on Day 6 of pregnancy. Experimental manipulation of the hormonal environment can cause delayed implantation as previous studies from this and other laboratories have shown(1). Implantation is delayed in the pregnant progesterone-treated ovariectomized (2,3) or hypophysectomized(3) rat. The anterior pituitary gland of the rat, when autografted to the kidney capsule, secretes abundant luteotrophin (Prolactin) with relatively little or no gonadotrophin release(4,5), and if the autograft is performed early in pregnancy, nidation is delayed(6). Administration of a single, small dose of estrogen initiates implantation and subsequent embryonic development in each of these experimental situations. It has been assumed that, since estrogen initiates implantation during the pe-

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